

# Proteasome-mediated degradation of I $\kappa$ B $\alpha$ and processing of p105 in Crohn disease and ulcerative colitis

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**Enhanced NF- $\kappa$ B activity is involved in the pathology of both forms of inflammatory bowel disease (IBD), Crohn disease (CD) and ulcerative colitis (UC). Here we analyzed the mechanism of proteasome-mediated NF- $\kappa$ B activation in CD and UC. Our studies demonstrate that the subunit composition and the proteolytic function of proteasomes differ between UC and CD. High expression of the immunoproteasome subunits  $\beta$ 1i and  $\beta$ 2i is characteristic of the inflamed mucosa of CD. In line with this, we found enhanced processing of NF- $\kappa$ B precursor p105 and degradation of inhibitor of NF- $\kappa$ B, I $\kappa$ B $\alpha$ , by immunoproteasomes isolated from the mucosa of CD patients. In comparison with healthy controls and CD patients, UC patients exhibited an intermediate phenotype regarding the proteasome-mediated processing/degradation of NF- $\kappa$ B components. Finally, increased expression of the NF- $\kappa$ B family member c-Rel in the inflamed mucosa of CD patients suggests that p50/c-Rel is important for IFN- $\gamma$ -mediated induction of immunoproteasomes via IL-12-driven Th1 responses. These findings suggest that distinct proteasome subunits influence the intensity of NF- $\kappa$ B-mediated inflammation in IBD patients.**

## Introduction

*Inflammatory bowel disease* (IBD) is a term that refers to 2 major chronic intestinal disorders of unknown etiology, Crohn disease (CD) and ulcerative colitis (UC). Genetic and environmental factors, including intestinal microflora, are believed to play a role in the development of IBD (1–4). Although both disease forms share some clinical and pathological features, the mechanisms underlying inflammation differ between CD and UC. While CD is associated with increased production of the Th1 cytokines IL-12, IFN- $\gamma$ , and TNF- $\alpha$ , UC is characterized by enhanced secretion of the Th2 cytokines IL-5 and IL-13 (5–10).

Aberrant activation of the transcription factor NF- $\kappa$ B controls the expression of many genes of inflammatory cytokines involved in the pathogenesis of IBD (11–15). In vertebrates, the NF- $\kappa$ B family consists of RelA (p65), NF- $\kappa$ B1 (p50/p105), NF- $\kappa$ B2 (p52/p100), c-Rel, and RelB. In many cell types, the most abundant form of NF- $\kappa$ B is the p50/p65 heterodimer, which remains in an inactive state in the cytoplasm, forming a ternary complex with the inhibitory protein inhibitor of NF- $\kappa$ B, I $\kappa$ B $\alpha$ . Upon stimulation, I $\kappa$ B $\alpha$  is rapidly phosphorylated, ubiquitinated, and subsequently degraded by proteasomes, allowing translocation of p50/p65 heterodimers into the nucleus (16–21). This results in activation of genes related to inflammation and proliferation. Another member of the NF- $\kappa$ B family is c-Rel, which is known to specifically regulate IL-12 production and consequently Th1 cell differentiation (22, 23).

Proteasomes are involved in 2 essential steps of NF- $\kappa$ B p50/p65 activation: generation of p50 from its precursor p105 and

degradation of I $\kappa$ B $\alpha$  (24–26). In cells, 26S proteasomes are responsible for the selective degradation of polyubiquitinated protein substrates. These multicatalytic enzymes consist of two 19S regulatory subunits and the catalytic 20S core complex, which is composed of 4 heptameric rings (27). Upon stimulation with type I and II interferons, the catalytic constitutive subunits  $\beta$ 1,  $\beta$ 2, and  $\beta$ 5 are replaced by the inducible  $\beta$ 1i (LMP2),  $\beta$ 5i (LMP7), and  $\beta$ 2i (MECL-1) subunits, leading to the formation of immunoproteasomes, which are characterized by enhanced substrate cleavage (28–32).

Here we isolated proteasomes from intestinal tissues of UC and CD patients and analyzed their subunit composition as well as their ability to degrade I $\kappa$ B $\alpha$  and to process p105 in vitro. Our results demonstrate that inflammation induces upregulation of immunoproteasomes in the inflamed mucosa of CD patients and, to a lesser extent, UC patients. Further, we show that processing of p105 and degradation of I $\kappa$ B $\alpha$  is most enhanced by proteasomes derived from CD patients. This correlates with increased amounts of NF- $\kappa$ B in the epithelium and lamina propria of these patients. Finally, our studies suggest that in CD patients, high expression of c-Rel is responsible for immunoproteasome upregulation via IL-12-induced IFN- $\gamma$  secretion.

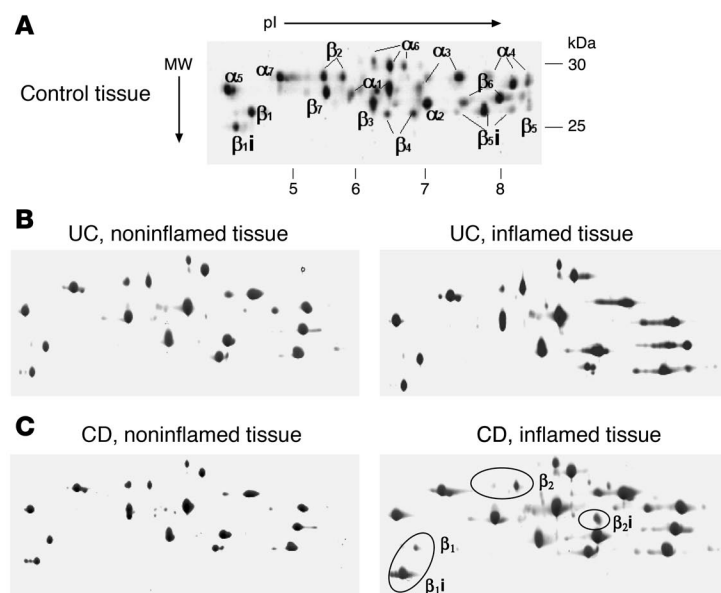
## Results

**Structure and enzymatic activity of 20S proteasomes in IBD patients.** We examined the subunit composition of 20S proteasomes purified from healthy intestinal tissues by 2D gel electrophoresis (2-DE) and subsequently identified proteins by matrix-assisted laser desorption/ionization mass spectrometry (MALDI-MS). More than 70 protein spots representing all 20S proteasome subunits and their isoforms in humans were identified (Figure 1A). We have recently shown that different mouse tissues reveal a characteristic pattern of 20S proteasomes (33). We observed that the  $\beta$ 5i immu-

**Nonstandard abbreviations used:** CD, Crohn disease; 2-DE, 2D gel electrophoresis; IBD, inflammatory bowel disease; LCL, lymphoblastoid cell line; MALDI, matrix-assisted laser desorption/ionization; MS, mass spectrometry; UC, ulcerative colitis.

**Conflict of interest:** The authors have declared that no conflict of interest exists.

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**Figure 1**

Subunit composition of proteasomes isolated from the colon of control patients and patients with IBD. Purified 20S proteasomes from the colonic mucosa were separated by 2-DE, and spots were identified by MALDI-MS. **(A)** 20S proteasome subunit pattern of control patients ( $n = 6$ ); lines indicate different isoforms of individual subunits. **(B and C)** Subunit composition of 20S proteasomes derived from noninflamed and inflamed mucosa of the colon from UC patients (UC, noninflamed tissue,  $n = 5$ ; UC, inflamed tissue,  $n = 5$ ) and CD patients (CD, noninflamed tissue,  $n = 5$ ; CD, inflamed tissue,  $n = 5$ ). Circles indicate increased expression of immunosubunits in inflamed mucosa of CD. Shown are representative 2-DE gels of 5 independent experiments.

nosubunit was constitutively expressed in the human colon, in contrast with the mouse colon.

Analysis of the impact of inflammation on the structure of 20S proteasomes revealed only slight differences in proteasome subunit composition between inflamed and noninflamed colon tissues of UC patients and controls (Figure 1, A and B). In contrast, strong upregulation of the immunosubunits  $\beta 1i$  and  $\beta 2i$  was found in the inflamed mucosa of CD patients (Figure 1C and Supplemental Table 1; supplemental material available online with this article; doi:10.1172/JCI28804DS1). To investigate proteolytic activities, we tested 20S proteasomes purified from inflamed colonic mucosa of CD and UC patients and controls for the turnover of fluorogenic substrates, representing trypsin-, chymotrypsin-, and caspase-like activities. CD patients revealed strong chymotrypsin-like activity while UC patients exhibited moderate levels that were marginally enhanced as compared with those of controls. The trypsin-like activity, which is attributed to  $\beta 2$  and  $\beta 2i$  subunits, was similar in all groups. Furthermore, proteasomes of CD patients showed reduced caspase-like activity as compared with UC patients and controls. (Figure 2, A–C). The hydrolytic activity against these substrates strongly depends on the content of immunosubunits. As reported by Groll and Huber (34), the replacement of constitutive subunit  $\beta 1$  by the immunosubunit  $\beta 1i$  results in reduced caspase-like, but enhanced chymotrypsin-like, activity. Consistent with this, the densitometric analysis of Western blots revealed low expression of  $\beta 1$  in the mucosa of CD patients compared with UC and control patients (Figure 2, D and E). This finding demonstrates that inflammation strongly affects the proteasome composition in CD but not UC patients.

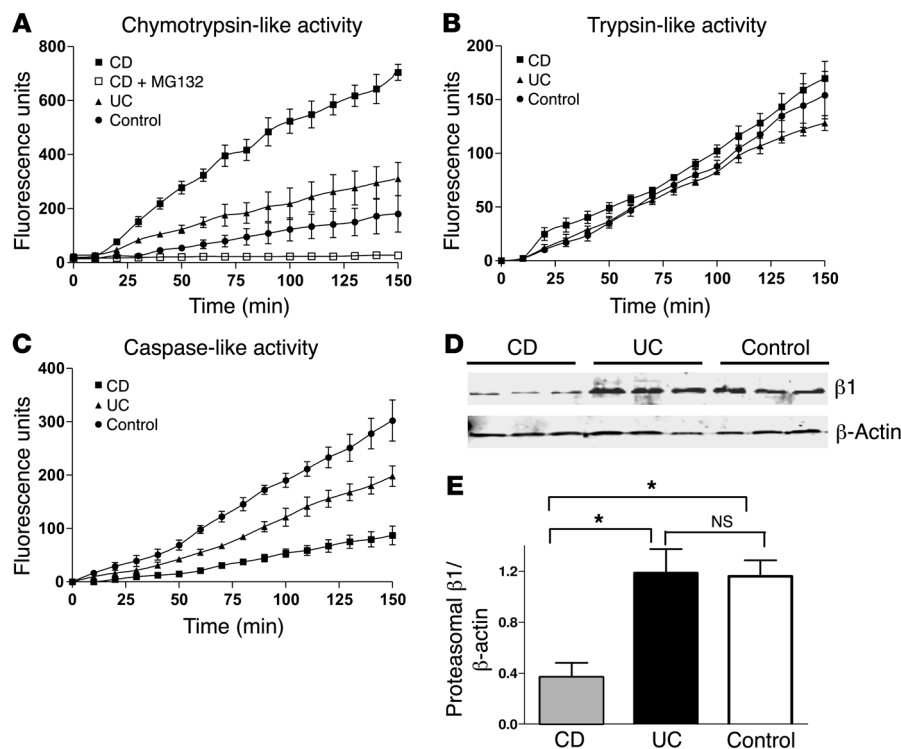
**Induction of immunoproteasomes in the intestinal mucosa and epithelial cells.** In order to analyze the distribution of immunoproteasomes, we stained tissue sections of IBD patients for the expression of the immunosubunit  $\beta 2i$ . Immunofluorescence studies revealed strong expression of the  $\beta 2i$  subunit, predominantly in the epithelium and cells of the lamina propria, in the terminal ileum and colon of CD patients. The expression of  $\beta 2i$  in the inflamed colonic epithelium of UC patients was weaker than in CD patients despite similar histological scores of CD and UC patients. No staining for

$\beta 2i$  was detectable in tissue sections of the terminal ileum or colon of healthy controls (Figure 3).

To determine signals required for the induction of immunosubunits in epithelial cells, we performed RT-PCR analyses of  $\beta 1i$ ,  $\beta 2i$ , and  $\beta 5i$  in Caco-2 cells stimulated with either different amounts of LPS or the inflammatory cytokines IFN- $\gamma$  and TNF- $\alpha$ . While stimulation with LPS or TNF- $\alpha$  exhibited no effects, IFN- $\gamma$  stimulation resulted in upregulation of immunosubunits that was not further enhanced by TNF- $\alpha$  (Figure 4). These results confirm previous findings (32) and suggest that immunoproteasome expression in the gut epithelium is induced by IFN- $\gamma$  but not by TNF- $\alpha$  or LPS.

**Kinetics of NF- $\kappa$ B p105 processing by 26S proteasomes of IBD patients.** We analyzed the effects of different proteasome subunit composition on processing of the NF- $\kappa$ B precursor p105 to p50, which can act either as p50/p65 and p50/c-Rel heterodimers or a p50/p50 homodimer. Cell lysates from the intestinal mucosa of IBD patients and controls were incubated with in vitro-translated p105 in the presence or absence of ATP. The kinetics of p105 processing demonstrates that the half-life of p105 was approximately 8 minutes and 40 minutes in the presence of proteasomes from CD or UC patients, respectively. After 10 minutes, p105 was almost completely processed by 26S proteasomes from CD patients while proteasomal processing was still incomplete after 120 minutes in UC patients and controls (Figure 5, A and B). Accordingly, the half-life of p105 in UC patients and controls was increased 5- or 12-fold compared with that of CD patients (Figure 5 D). In vitro processing of p105 was strictly ATP dependent, demonstrating that generation of p50 from p105 was mediated by 26S proteasomes (Figure 5C). To exclude effects of contaminating proteases, we performed in vitro processing of p105 in the presence of the proteasome inhibitor MG132 and found complete inhibition by 10  $\mu$ M MG132 (data not shown).

**Kinetics of I $\kappa$ B $\alpha$  degradation by 20S proteasomes of IBD patients.** Degradation of I $\kappa$ B $\alpha$  is mediated by both 26S and 20S proteasomes (35). We thus compared the efficiency of 20S proteasomes isolated from inflamed mucosa of IBD patients and mucosa of healthy controls in degrading in vitro-translated I $\kappa$ B $\alpha$ , which is necessary for the activation of the NF- $\kappa$ B p50/p65 complex.



**Figure 2**

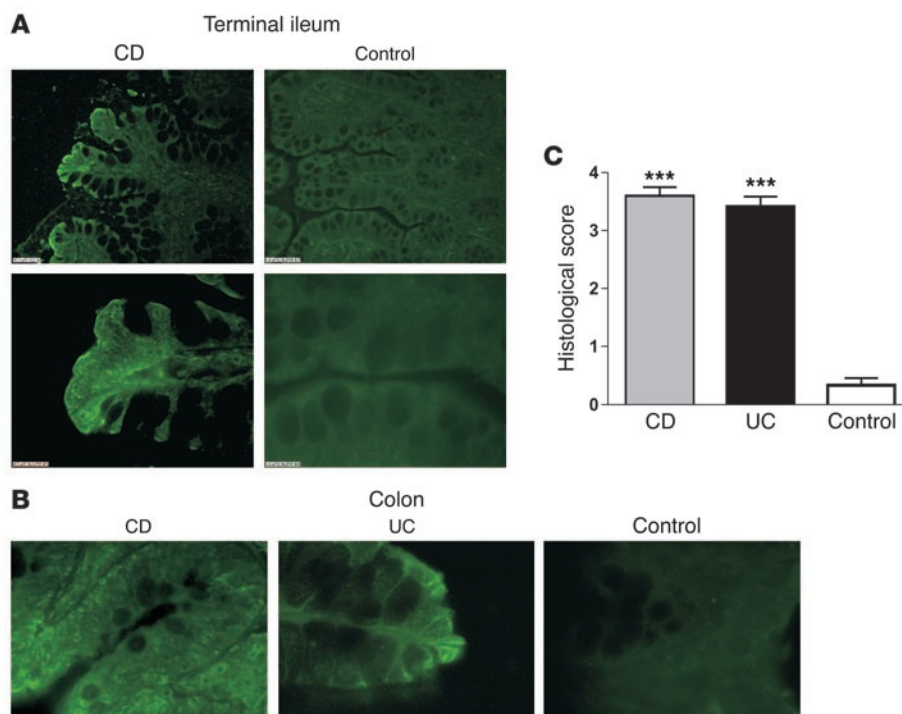
Proteolytic activity of proteasomes in IBD patients. (A–C) Enzymatic activity of 20S proteasomes purified from colonic mucosa of CD patients (filled squares), UC patients (triangles), and control patients (circles) was measured by cleavage of fluorogenic peptide substrates. Control reaction was performed in the presence of the proteasome inhibitor MG132 (open squares). Data are expressed as means  $\pm$  SEM. ( $n = 5$ ). Statistical significance was determined by 1-way ANOVA (chymotrypsin- and caspase-like activity,  $P < 0.001$ ; trypsin-like activity,  $P = 0.114$ ). (D) Western blot of lysates from inflamed intestinal mucosa of CD patients, UC patients, and controls was stained for the proteasomal immunosubunit  $\beta 1$  and  $\beta$ -actin. (E) Quantitative analysis of the proteasomal  $\beta 1/\beta$ -actin protein ratio was performed using AIDA Image Analyser software (version 4.0.3; Raytest). Data represent means  $\pm$  SEM; CD ( $n = 10$ ), UC ( $n = 10$ ), and controls ( $n = 9$ ).  $*P < 0.05$ , Student's  $t$  test ( $n = 9$ – $10$ ).

In contrast with very slow proteasomal degradation of  $\text{I}\kappa\text{B}\alpha$  in controls, the half-life of  $\text{I}\kappa\text{B}\alpha$  incubated with purified 20S proteasomes from CD patients was approximately 7 minutes. Proteasomes from UC patients revealed an intermediate activity, with an  $\text{I}\kappa\text{B}\alpha$  half-life of approximately 30 minutes (Figure 6A). Thus proteasomal degradation of the NF- $\kappa\text{B}$  inhibitory protein  $\text{I}\kappa\text{B}\alpha$  by CD patients was 4- and 10-fold faster compared with that

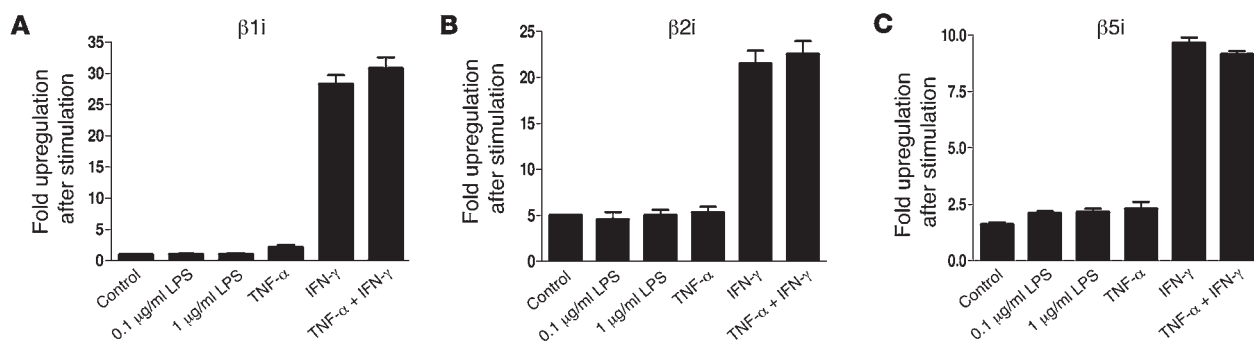
of UC patients and controls, respectively (Figure 6B). This finding demonstrates that increased expression of immunosubunits in 20S proteasomes is responsible for the rapid degradation of  $\text{I}\kappa\text{B}\alpha$  observed in CD patients. To further determine whether the observed effects were due to immunoproteasomes, experiments were repeated with purified 20S proteasomes from WT or mutant lymphoblastoid cell lines (LCLs) that either contain (LCL 721) or

**Figure 3**

Expression of immunoproteasomes in the intestinal mucosa of IBD patients. (A and B) Immunofluorescent staining of sections from the terminal ileum (A) and colon (B) of IBD patients and controls with an antibody to  $\beta 2i$ . Magnification,  $\times 200$  (A, upper panel);  $\times 1000$  (A, lower panel, and B). (C) Inflammation scores of patients shown in A and B (CD,  $n = 10$ ; UC,  $n = 8$ ; control,  $n = 10$ ;  $***P < 0.001$  compared with control).





**Figure 4**

Relative expression of proteasomal immunosubunits in Caco-2 cells. Differentiated Caco-2 cells were stimulated with LPS, TNF- $\alpha$ , and IFN- $\gamma$ , and the expression of  $\beta 1i$  (A),  $\beta 2i$  (B), and  $\beta 5i$  (C) was measured by real-time PCR. Data represent means  $\pm$  SEM ( $n = 3$ ).

lack immunoproteasomes (LCL 721.174). Whereas 20S proteasomes from LCL 721.174 cells showed high similarity to 20S proteasomes of control patients, immunoproteasome-rich LCL 721 cells displayed proteolytic activity resembling that of CD patients (Figure 6, C and D, and Supplemental Figure 3).

**Effects of a probiotic factor on human proteasomes and on nascent colitis in mice.** Recent data demonstrated that a soluble factor secreted by a mixture of probiotic bacteria (VSL#3) blocks degradation of I $\kappa$ B $\alpha$  and processing of the NF- $\kappa$ B precursor p105 by inhibiting the chymotrypsin-like activity of proteasomes in murine cells (36). To examine the effect of this inhibitor on human proteasomes, we preincubated 20S proteasomes isolated from inflamed intestines of CD patients with conditioned media from VSL#3 or *E. coli* strain Nissle 1917 and performed proteasome activity assays. We observed that the conditioned medium of VSL#3 but not of *E. coli* strain Nissle 1917 inhibited the chymotrypsin-like activity of immunoproteasomes from CD patients (Figure 7A). A synthetic proteasome inhibitor MG132 was used as a control. In addition, treatment of IL-10 KO mice with conditioned medium of VSL#3 before the onset of colitis prevented weight loss during the observation period of 3 weeks as compared with mock-treated mice (Figure 7B). These findings suggest that inhibition of the chymotrypsin-like activity of protea-

somes with nontoxic bacterial compounds attenuates the development of spontaneous colitis in mice.

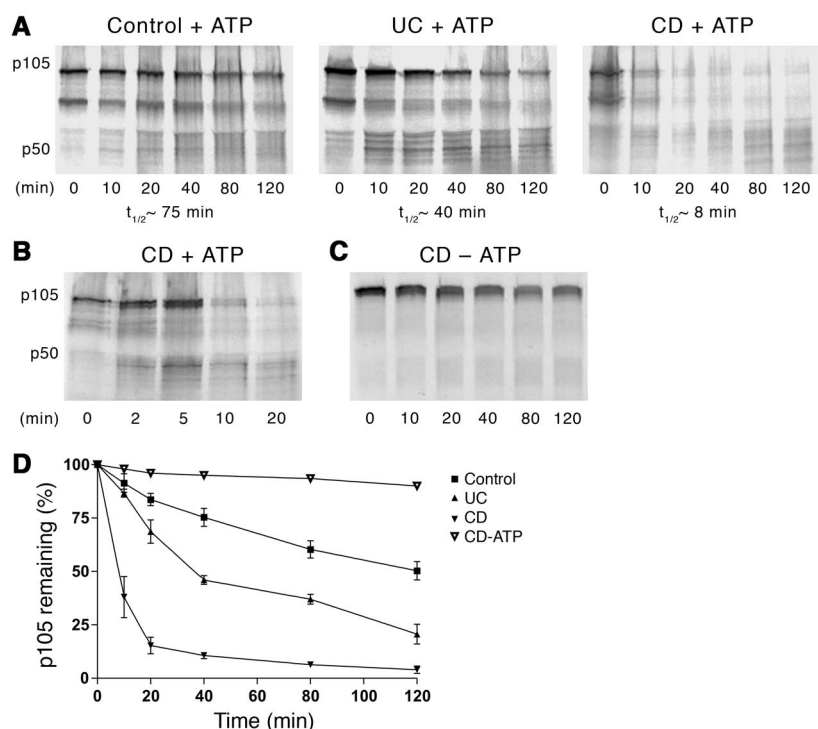
**Expression of NF- $\kappa$ B components and immunoproteasomes in IBD patients.**

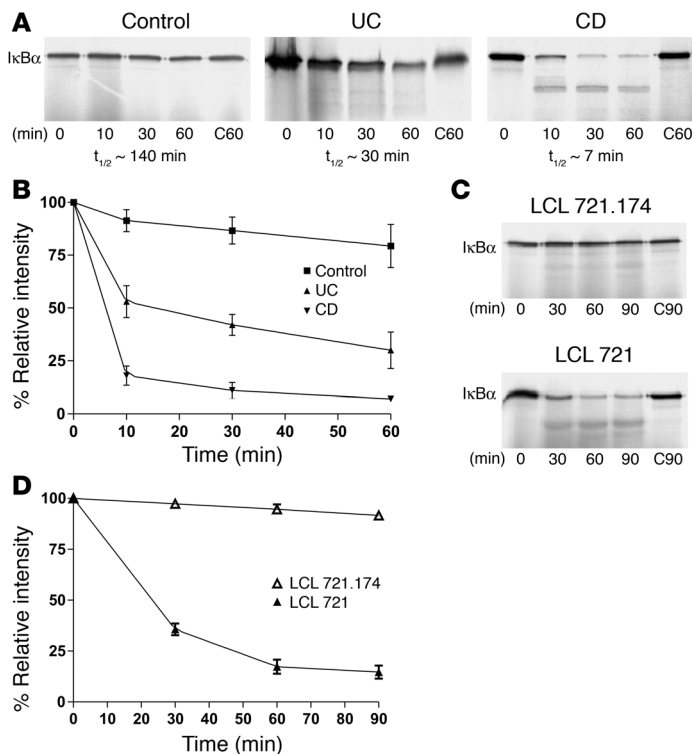
To determine whether enhanced expression of NF- $\kappa$ B components is also detectable in the inflamed intestinal mucosa of CD patients, we compared lysates of CD and UC patients as well as controls for expression of p105 and p50 in the cytosol and the abundance of nuclear p65 and c-Rel. Despite individual variations among patients, we found common patterns for CD and UC patients. Enhanced nuclear expression of NF- $\kappa$ B subunits p65 and c-Rel and very low amounts of p105 in the cytosol were characteristic for CD but not for UC patients and controls (Figure 8). These findings support our in vitro data on increased p105 processing due to immunoproteasomes and suggest that active NF- $\kappa$ B complexes p50/p65 and p50/c-Rel are predominant in the inflamed mucosa of CD patients.

Finally, we investigated whether enhanced activation of NF- $\kappa$ B correlates with increased expression of immunoproteasomes in

**Figure 5**

Proteasomal processing of NF- $\kappa$ B precursor protein p105. (A) Kinetics of processing of p105 by proteasomes from the colonic mucosa of IBD patients and controls (CD,  $n = 6$ ; UC,  $n = 5$ ; controls,  $n = 5$ ).  $^{35}\text{S}$ -labeled in vitro-translated p105 (Supplemental Figure 1) was incubated with cytosolic lysates of colonic tissues in the presence of 10 mM ATP. A representative autoradiogram is shown for each group of patients. (B) Detailed analysis of processing of p105 during first 20 minutes by lysates from CD patients. (C) Processing of p105 in the absence of ATP by lysates from CD patients. (D) Quantitative analysis of autoradiograms, showing significant differences in the processing of p105 among CD patients, UC patients, and controls. Data represent mean values  $\pm$  SEM.  $P < 0.0001$  by 1-way ANOVA.



**Figure 6**

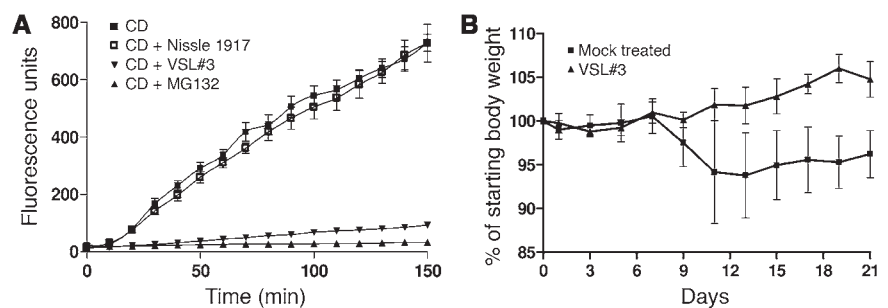
Effect of immunoproteasomes on degradation of IκBα. **(A)** Kinetics of IκBα degradation by 20S proteasomes purified from colonic mucosa of patients with IBD and controls (CD,  $n = 5$ ; UC,  $n = 5$ ; controls,  $n = 5$ ). IκBα was in vitro translated in the presence of  $^{35}$ S-methionine (Supplemental Figure 2) and incubated with isolated 20S proteasomes for indicated times. C60 represents a control incubated for 60 minutes without 20S proteasomes with an equivalent amount of IκBα. **(B)** Quantitative evaluation of IκBα degradation by 20S proteasomes isolated from patients with IBD and controls in the absence of ATP.  $P < 0.0001$  (by 1-way ANOVA);  $n = 5$ . **(C)** Analysis of degradation of in vitro-translated IκBα by 2 different subsets of 20S proteasomes. Data from 1 representative experiment are shown for purified constitutive proteasomes (LCL 721.174;  $n = 3$ ) and immunoproteasomes (LCL 721;  $n = 3$ ). Lack of β1i and β5i in LCL 721.174 excludes the incorporation of immunosubunits in 20S proteasomes. **(D)** Quantification of IκBα degradation by 20S proteasomes isolated from LCL 721 and LCL 721.174 cells ( $n = 3$ ).

vivo. We stained control and inflamed tissues of terminal ileum and colon for expression of β2i and active NF-κB p65 using antibodies specific for the nuclear localization sequence of p65, which can only be detected after activation of NF-κB. Double immunofluorescence revealed colocalization of activated p65 and proteasomal immunosubunit β2i mainly in the intestinal mucosa of CD patients, suggesting the impact of immunoproteasomes on activation of NF-κB in vivo (Figure 9). We here propose a mechanism for enhanced NF-κB activity in CD mediated by immunoproteasomes: Early inflammatory signals trigger expression of c-Rel, which leads to increased IL-12 expression and a strong Th1-mediated T cell response. High levels of IFN-γ induce immunoproteasomes, which are involved in enhanced activation of NF-κB and exacerbation of inflammation (Figure 10).

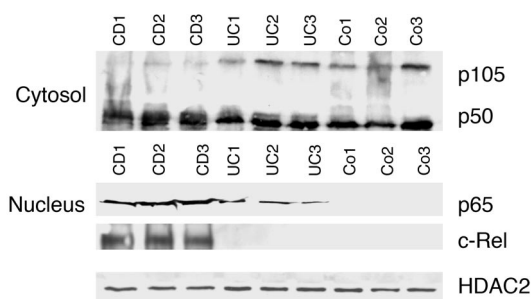
## Discussion

In this study, we report that differences in proteasome subunit composition in the inflamed intestines of UC and CD patients have an impact on the level of NF-κB activation. Increased processing of p105 and rapid degradation of IκBα by immunoproteasomes in CD patients may be responsible for enhanced expression of inflammatory genes regulated by p50/c-Rel and p50/p65 heterodimers. Although NF-κB p65 is involved in the pathogenesis of UC and CD, our data show that the abundance and mechanism of induction differ between these disease forms.

IFN-γ stimulation induces the incorporation of β1i, β2i, and β5i immunosubunits into the 20S proteasome; of these immunosubunits, β1i and β2i were selectively upregulated in the inflamed colon, and that upregulation was even more pronounced in the terminal ileum of CD patients. The β5i subunit is constitutively expressed in the human intestine independent of any signs of inflammation, in contrast with that of mice. We speculate that constitutive expression of β5i represents an adaptation of the human intestine in order to rapidly induce all 3 immunosubunits, as β5i has been shown to catalyze the assembly of immunoprotea-

**Figure 7**

Proteasome inhibitor secreted by probiotic bacteria inhibits the chymotrypsin-like activity of 20S proteasomes isolated from CD patients and prevents nascent colitis in IL-10 KO mice. **(A)** The chymotrypsin-like activity of 20S proteasomes (2 μg) purified from CD patients ( $n = 6$ ) was measured in the presence (50 μl) or absence of conditioned media from probiotic bacteria (VSL#3) and *E. coli* strain Nissle 1917. As control, the synthetic proteasome inhibitor MG132 was used at a concentration of 10 μM. **(B)** IL-10 KO mice were treated before the onset of colitis either with PBS- (control) or VSL#3-conditioned medium. Treatment started at the age of 10 weeks and was performed for 3 weeks by giving either 500 μl PBS or VSL#3 intragastrically every other day. Weight was monitored for both groups ( $n = 8$ ).

**Figure 8**

Cytosolic and nuclear extracts obtained from colonic specimens analyzed by Western blot using antibodies directed against cytosolic p105 and p50 as well as nuclear p65 and c-Rel proteins (CD,  $n = 9$ ; UC,  $n = 9$ ; controls,  $n = 10$ ). HDAC2 was used as loading control for nuclear extracts.

somes (37). Incorporation of the IFN- $\gamma$  inducible subunits  $\beta 1i$  and  $\beta 2i$  in proteasomes of CD patients altered their proteolytic activity. While trypsin-like activity remained unchanged, immunoproteasomes of CD patients revealed enhanced chymotrypsin-like and reduced caspase-like activity as compared with UC patients and controls. However, it must be mentioned that, compared with controls, increased chymotrypsin- and decreased caspase-like activities were also found in UC, although the differences were less prominent than in CD patients.

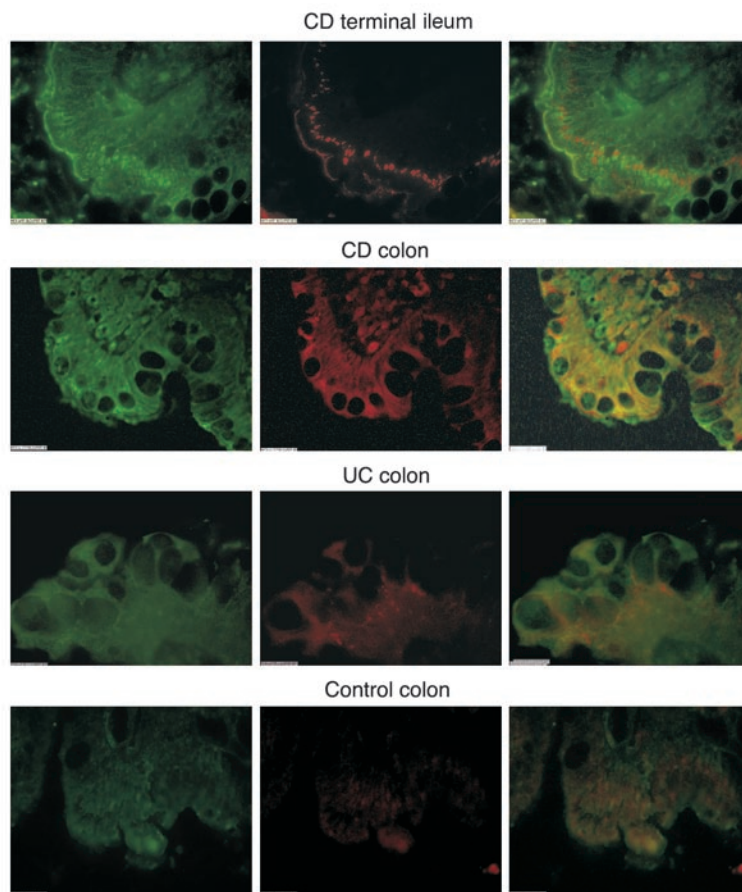
We obtained evidence for the involvement of immunoproteasomes in enhanced NF- $\kappa$ B activation when we showed that *in vitro* processing of NF- $\kappa$ B p105 and degradation of I $\kappa$ B $\alpha$  were accelerated in CD patients. Compared with healthy controls and UC patients, proteasomal processing of p105 in the inflamed colonic mucosa of CD patients was 12- or 5-fold faster, respectively. Kinetic studies further demonstrated that the half-lives of I $\kappa$ B $\alpha$  degraded by purified 20S proteasomes from CD patients was shortest (7 minutes), followed by those of UC patients (30 minutes), and was longest in controls (140 minutes). Together with the data from cell lines demonstrating that degradation of I $\kappa$ B $\alpha$  is directly linked to the expression of proteasomal immunosubunits, these results confirm the profound effect of different proteasome subsets on NF- $\kappa$ B activation. Thus the hierarchy of immunosubunit expression in IBD patients is reflected by their function.

An explanation for high immunosubunit expression in CD patients may be provided by the strong expression of c-Rel in the inflamed mucosa of CD patients. Increased amounts of p50 and c-Rel suggest formation

of p50/c-Rel complexes, which were previously shown to regulate IL-12 expression (22, 23). IL-12 is a key cytokine for Th1 cell polarization. This in turn initiates IFN- $\gamma$  production and thus controls the induction of immunoproteasomes. The distinction of UC and CD patients with respect to c-Rel expression in the inflamed mucosa confirms previous observations that CD is associated with a Th1 cell response whereas UC exhibits more of a Th2-like cytokine pattern (1, 5). In accordance with this, treatment of Th1-mediated colitis in animals and CD patients with anti-IL-12 and anti-TNF- $\alpha$  antibodies has been shown to improve inflammatory reactions (38, 39).

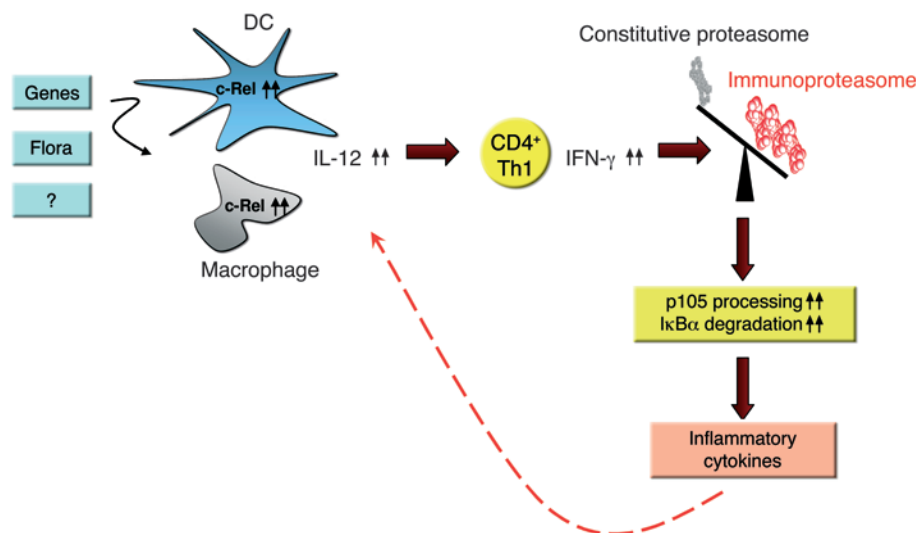
The involvement of proteasomes in intestinal inflammation has also been emphasized by studies from Montelone et al. (40, 41). In addition to the direct activation of NF- $\kappa$ B, proteasomes also control inflammation indirectly by degrading Smad7, an inhibitor of TGF- $\beta$ 1 signaling. In normal tissues, TGF- $\beta$ 1 signaling negatively regulates NF- $\kappa$ B activation by induction of I $\kappa$ B $\alpha$ . However, in the inflamed gut of IBD patients, high expression of Smad7, an intracellular inhibitor of TGF- $\beta$ 1 signaling, allows inflammation to proceed unchecked. Posttranscriptional acetylation of Smad7 in IBD patients is believed to be responsible for its resistance to proteasomal degradation that normally occurs in the healthy gut.

Many of the antiinflammatory drugs used to treat experimental colitis are based on the inhibition of NF- $\kappa$ B activation, which underlines its importance as a pathogenic factor. Blocking NF- $\kappa$ B activity with antisense oligonucleotides specific for p65 and decoy oligonucleotides targeting the NF- $\kappa$ B consensus binding sequence has been shown to be a potent method to terminate or even reverse established inflammation (11, 42). As this might be the most

**Figure 9**

Double immunofluorescence of proteasomal immunosubunit  $\beta 2i$  and NF- $\kappa$ B subunit p65 in the terminal ileum and colon of IBD patients and controls. Magnification,  $\times 1,000$ . Increased expression of NF- $\kappa$ B subunit p65 colocalizes with enhanced expression of the  $\beta 2i$  subunit in intestinal epithelial cells and cells of the lamina propria (green fluorescence,  $\beta 2i$ ; red fluorescence, p65). NF- $\kappa$ B was stained with a primary antibody that exclusively recognizes activated p65. Data represent 1 of 8–10 patients per group.



**Figure 10**

Schematic overview of enhanced immunoproteasome-mediated NF- $\kappa$ B activation and consequent exacerbation of inflammation in CD patients. Unknown initial inflammatory events induce expression of c-Rel in the intestinal mucosa and a strong Th1 cell response via IL-12. High amounts of IFN- $\gamma$  trigger generation of immunoproteasomes, which enhances NF- $\kappa$ B activation, thus perpetuating the inflammatory process.

effective method to rapidly treat established IBD, it remains to be determined whether a complete blockade of major components of NF- $\kappa$ B is also suitable for long-term treatment. Since controlled inflammation represents a crucial defense mechanism against various pathogens, complete blockade of the inflammatory response could strongly affect antimicrobial resistance in these patients. In this study, we show that a proteasome inhibitor secreted by probiotic bacteria specifically inhibits the chymotrypsin-like activity of 20S proteasomes from CD patients and attenuates the development of spontaneous colitis in IL-10 KO mice. However, it remains to be determined whether specific inhibition of immunosubunits  $\beta$ 1i and  $\beta$ 2i can block excessive NF- $\kappa$ B activation without ablating the NF- $\kappa$ B-mediated inflammatory reactions required for protection against pathogens.

## Methods

**Patients.** Human tissue was obtained from colonic and small intestinal specimens immediately after intestinal surgery performed at the Charité University Hospital in Berlin. All patients provided informed consent. This study was approved by the local ethics committee of the Charité Universitätsmedizin Berlin. A total of 25 patients with active CD, 20 patients with active UC, and 28 tumor patients acting as controls were investigated. In 9 CD patients, the primary site of disease was the colon, and in 16 patients, both terminal ileum and colon were involved. At the time of sample collection, 15 patients of the CD group were receiving corticosteroids, 4 were receiving azathioprine, 3 were receiving 5-ASA, and 4 were on no medications. In the UC group, 11 patients were receiving corticosteroids, 4 were receiving azathioprine, and 6 were not treated with any immunosuppressive drug. Mucosal tissue layers were mechanically dissected from the underlying submucosa, immediately frozen in liquid nitrogen, and stored at  $-196^{\circ}\text{C}$  until use. The degree of inflammation in the surgical specimens was evaluated using a scoring system for UC as follows: 0, no inflammation; 1, mild, chronic, inactive inflammation (architectural changes, chronic inflammatory infiltrate); 2, mild, active inflammation (lamina propria neutrophils); 3, moderate, active inflammation (crypt destruction); 4, severe, active (erosions or ulcerations) inflammation. The scoring system for CD was similar: 0, normal; 1, mild edema and inflammation in the lamina propria; 2, crypt abscesses and moderate lamina propria inflammation; 3, more severe inflammation with destructive crypt abscesses plus or minus granulomata; 4, fulminant inflammation with ulcerations or fissures.

**Mice.** C57BL/6 mice and IL10 $^{-/-}$  mice on C57BL/6 background were bred in the animal facilities of the Max Planck Institute for Infection Biology under specific pathogen-free conditions. Experiments using animals were approved by the local ethics committee of the Charité Universitätsmedizin Berlin and by the German authorities (Landesamt für Gesundheit und Soziales Berlin).

**Cell lines.** LCL 721 and LCL 721.174 cells were described previously (43). Cell lines were provided by V. Cerundolo (John Radcliffe Hospital, Oxford, United Kingdom). Caco-2 cells were purchased from ATCC.

**Preparation of bacteria-conditioned media.** Probiotic bacterial mixture VSL#3 (VSL Pharmaceuticals) and *E. coli* strain Nissle 1917 were grown to a concentration of approximately  $10^{14}$  bacteria in phenol red-free RPMI 1640 medium (Invitrogen) for 16 hours and then centrifuged. Supernatants were passed through a 0.22- $\mu\text{m}$  filter and used in proteasome activity assays (50  $\mu\text{l}$ ) and oral treatment of mice (500  $\mu\text{l}$ ).

**Purification of 20S proteasomes.** The 20S proteasomes were purified as previously described (33). For preparation of proteasomes from the small intestine, the 3-fold higher concentration of protease inhibitors was used. The purity of 20S proteasome preparations was tested by SDS-PAGE.

**2-DE and MALDI.** The 20S proteasome preparations were desalted and concentrated by trichloroacetic acid precipitation. Pellets were resolubilized in 2-DE sample buffer and subjected to electrophoresis. For resolution of 20 proteasomal subunits, isoelectric focusing by carrier ampholytes was combined with SDS-PAGE. Between 20 and 80  $\mu\text{g}$  proteins were applied to the anodic side of a carrier ampholyte isoelectric focusing gel. In the second dimension, the proteins were separated in SDS-PAGE gels (23  $\times$  30 cm and 7  $\times$  8 cm) and stained by Coomassie Brilliant Blue G250 and silver, respectively. For mass spectrometric identification, each individual spot detected on the 2D gel was cut out, digested by trypsin, desalted and concentrated by ZipTip (Millipore), and analyzed by MALDI-MS (4700 Proteomics Analyzer; Applied Biosystems). Finally, database searches were performed using combined peptide mass fingerprinting and MS/MS datasets.

**Proteasome activity assay.** Proteolytic activities of proteasomes were assayed with fluorogenic peptide substrates. Purified 20S proteasomes (1  $\mu\text{g}$ ) were added to the reaction buffer (50 mM Tris, pH 7.5, 10 mM NaCl, 30 mM KCl, 0.1 mM EDTA, and 20  $\mu\text{M}$  substrate), and fluorescence was detected by fluorescence microtiter reader Fluoroskan Ascent (Thermo Labsystems; Thermo Electron Corp.) at 355-nm excitation/460-nm emission for 7-amino-4-methylcoumarin (AMC). Caspase-like activity was assayed with Z-Leu-Leu-Glu-AMC, chymotrypsin-like activity with Suc-Leu-Leu-



Val-Tyr-AMC, and trypsin-like activity with Bz-Val-Gly-Arg-AMC as substrates. Inhibitor studies were carried out by assaying proteolytic activities of the proteasomes after preincubation with MG132 at a concentration of 10  $\mu$ M. For kinetic assays, fluorescence was measured every 10 minutes and data were analyzed using Ascent software, version 2.6 (Thermo Labsystems; Thermo Electron Corp.). All experiments were performed at least 3 times.

**Western blot analysis.** Proteasomal subunit  $\beta$ 1 was detected using rabbit anti-human antibody (Affinity Research Products). Nuclear NF- $\kappa$ B subunit p65 was stained with a rabbit anti-human NF- $\kappa$ B/p65 antibody (Santa Cruz Biotechnology Inc.) and cytosolic p50 and its precursor p105 with a rabbit anti-human NF- $\kappa$ B/p50 and p105 antibody (eBioscience). The specific antibody against NF- $\kappa$ B subunit c-Rel was purchased from Rockland Immunochemicals. As control for protein amounts, a monoclonal anti-human  $\beta$ -actin antibody (Sigma-Aldrich) and a rabbit anti-human histone deacetylase (HDAC2) antibody (Sigma-Aldrich) were used.

**RT-PCR.** Total RNA from differentiated Caco-2 cells was extracted with TRIzol (Invitrogen) and treated with RNase-free DNase to remove contaminating genomic DNA from the sample. RNA quality was checked with the 2100 Bioanalyzer (Agilent Technologies). After reverse transcription, real time-PCR was performed with 2 $\times$  SYBR Green PCR Master Mix (Applied Biosystems) using an ABI Prism 7000 Sequence Detection System (Applied Biosystems).

**Immunofluorescence studies.** Immunofluorescence studies were performed using intestinal cryosections. Normal goat serum was applied to block nonspecific protein binding. As primary antibodies, a polyclonal rabbit antibody against human  $\beta$ 2i (Affinity Research Products) and a monoclonal mouse antibody against NF- $\kappa$ B p65 subunit (Chemicon International) were used. This was followed by incubation with secondary IgG antibody conjugated with Cy2 and Cy3, respectively.

**Preparation of cytosolic and nuclear extracts from intestinal tissue.** Ground intestinal tissue was immersed in ice-cold lysis buffer (10 mM HEPES, pH 7.9, 10 mM KCl, 1.5 mM MgCl<sub>2</sub>, 1 mM DTT, 1.5 mM PMSF, 20 mM NaF, 200  $\mu$ M Na<sub>3</sub>VO<sub>4</sub>, and protease inhibitor cocktail). After keeping the sample on ice for 15 minutes, 25  $\mu$ l 10% NP-40 was added per 1 ml homogenate. Further samples were incubated on ice for another 20 minutes and centrifuged at 10,000 *g* for 2 minutes at 4°C. Supernatants were used as cytosolic

extracts. Pelleted nuclei were washed with PBS and resuspended in extraction buffer (20 mM HEPES, pH 7.9, 420 mM NaCl, 1.5 mM MgCl<sub>2</sub>, 0.2 mM EDTA, 1 mM DTT, 1.5 mM PMSF, 20 mM NaF, 200  $\mu$ M Na<sub>3</sub>VO<sub>4</sub>, and protease inhibitor cocktail). Finally, nuclear suspensions were centrifuged at 10,000 *g* for 30 minutes at 4°C to collect the supernatants containing nuclear protein extracts.

**In vitro processing of p105 and I $\kappa$ B $\alpha$  degradation assay.** Coding sequences of p105 and I $\kappa$ B $\alpha$  were cloned into the expression vector pcDNA3 harboring FLAG epitope, respectively. These constructs were subjected to a reticulocyte lysate-based coupled transcription/translation in vitro system (Promega) in the presence of <sup>35</sup>S-methionine. Afterward, <sup>35</sup>S-labeled p105 and I $\kappa$ B $\alpha$  proteins were used as substrates for in vitro processing/degradation assay. The <sup>35</sup>S-labeled p105 was incubated with cell lysates (80  $\mu$ g of protein) and <sup>35</sup>S-labeled I $\kappa$ B $\alpha$  with 2  $\mu$ g purified 20S proteasomes from the intestinal mucosa of IBD patients and controls, respectively. The processing/degradation was measured at 37°C in the absence or presence of 10 mM ATP. Control reactions contained 10  $\mu$ M MG132 (Sigma-Aldrich). The samples were quantified using LAS-3000 imaging system (Fujifilm).

**Statistics.** Data were analyzed using 2-tailed Student's *t* test. For analyzing multiple groups, 1-way ANOVA was used, and results were considered significant at *P*  $\leq$  0.05.

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